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(54) Title: MANIPULATION OF MITOTICALLY ACTIVE CELLS OF THE HIPPOCAMPAL REGION OF THE BRAIN

(57) Abstract

A method is disclosed for the *in vivo* proliferation of a multipotent neural stem cell located in the hippocampal region of a mammal. The method comprises administering at least one growth factor to the hippocampal region which induces the proliferation of the stem cell to produce stem cell progeny that are capable of differentiating into neurons and glial cells. The method provides treatments for neurological diseases, injuries, or disorders which affect the hippocampal region of the brain, such as stroke.

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MANIPULATION OF MITOTICALLY ACTIVE CELLS OF THE HIPPOCAMPAL REGION OF THE BRAIN

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Field of the Invention:

This invention relates to the *in vitro* and *in vivo* manipulation of the population of mitotically active cells which have been identified in the hippocampal region of the adult brain. More particularly, this invention is related to a method of directly manipulating the endogenous precursor cells of the hippocampal formation, *in vivo*, to induce them to divide, differentiate and migrate so as to augment hippocampal cell numbers in order to increase or alter synaptic efficacy or to replace dysfunctional hippocampal cells, or those lost to injury or disease.

Background of the Invention:

It is generally accepted that the adult brain is unable to repair itself following an injury or a damaging disease. Until recently, it was thought that the adult brain lacked stem cells capable of proliferating new cells to repair damaged neural tissue. Reynolds and Weiss discovered that a small percentage of undifferentiated cells isolated from the adult mammalian central nervous system (CNS) can be induced to proliferate *in vitro* and amongst their progeny are cells which can be induced to differentiate into neurons, astrocytes, and

oligodendrocytes (Science vol. 255, (March 27, 1992) pp. 1707-1710. This small percentage of undifferentiated neural cells exhibit the three main defining characteristics of stem cells: they are undifferentiated cells capable of proliferation, self-maintenance (i.e. capable of dividing without limit) and the production of a large number of differentiated, functional progeny. (See Potten & Loeffler, Development 110 (1990) pp. 1001-1020). Under suitable culture conditions, such as those disclosed in published PCT Application Nos. WO 93/01275, WO 94/10292, WO 94/16718 and WO 95/13364, the stem cells can be continuously proliferated and passaged resulting in large numbers of undifferentiated cells. Culture conditions can be changed to induce the differentiation of the stem cell progeny. The neural stem cells are multipotent because a single stem cell can produce progeny that differentiated into the three types of differentiated cells of the CNS: astrocytes (types I and II), oligodendrocytes, and neurons. The findings of Reynolds and Weiss raised the possibility that brain repair, following disease or accidental injury, could be feasible through the manipulation of the neural stem cell population, allowing afflicted patients to enjoy an improved quality of life.

The most common disease of the nervous system is cerebrovascular accident (CVA), commonly known as a "stroke." This condition, which is the result of a disruption, due to blockage or hemorrhage of the blood supply to the cortex, is one of the leading causes of chronic disability in the United States. The nature and severity of the disability in an individual is dependent on the regions of the brain involved and the severity of the CVA. CVA induced lesions frequently affect the hippocampal region of the brain, particularly the subfields of Ammon's Horn including the pyramidal layers of the CA1 and CA3 regions. Such lesions are manifested in impaired short term memory and an inability to learn.

It has been known for many years that a population of mitotically active cells in the mammalian dentate gyrus retain the ability to generate neurons and glia well into post-natal life. However, as no mitotically active cells were reported

outside the dentate gyrus, it was believed that mitotically active cells were absent from other regions of the adult hippocampus. As the hippocampal region is an integral component of memory and sensory integration and thus the ability to learn, it would be desirable to be able to induce precursor cells throughout the hippocampal region to generate new hippocampal cells to augment normal function or to repair a deficit caused by disease or injury. It would also be desirable to be able to genetically modify cells throughout the hippocampal region to induce them to produce gene products useful in the augmentation of normal function or in the treatment of disease or injury in the region. A method of screening the effects of drugs, growth factors, and other biological agents on the mitotically active cells of the hippocampal region and their progeny would also be useful in the development of therapies for use in the treatment of neural disorders such as stroke.

Detailed Description of the Invention:

We have identified a small population of previously unreported mitotically active cells located throughout all the subfields of the Ammon's horn region of the hippocampus and in other areas of the hippocampal region. The hippocampal region is described by K.Y. Reznikov (Adv. Anat. Embryol. cell. Biol. (1991) Vol. 122: ¶. 1-83). Labeling experiments demonstrate that these mitotically active cells differentiate into neurons and glia A similar population of cells, which has been found in other adult CNS regions such as the subependymal linings of ventricles and areas that correspond to the germinal matrices, including the amygdala, have been identified as neural stem cells. The mitotically active cells of the hippocampal region also exhibit stem cell characteristics. Accordingly, the procedures set forth in WO 93/01275, WO 94/10292 and WO 95/13364, with respect to the *in vitro* and *in vivo* proliferation and use of multipotent neural stem cells and their progeny are applicable to the mitotically active cells of the hippocampal region identified in the present application.

As used herein, the term "neural stem cell" refers to an undifferentiated neural cell that can be induced to proliferate using the methods of Reynolds and Weiss disclosed in the related applications referenced above. A neural stem cell is capable of self-maintenance, meaning that with each cell division, one daughter cell will also be a stem cell. The non-stem cell progeny of a neural stem cell are termed "progenitor cells." The progenitor cells generated from a single multipotent neural stem cell are capable of differentiating into neurons, astrocytes (type I and type II) and oligodendrocytes. Hence, the neural stem cell is "multipotent" because its progeny have multiple differentiative pathways.

The term "neural progenitor cell", as used herein, refers to an undifferentiated cell derived from a neural stem cell, and is not itself a stem cell. Some progenitor cells can produce progeny that are capable of differentiating into more than one cell type. For example, an O-2A cell is a glial progenitor cell that gives rise to oligodendrocytes and type II astrocytes, and thus could be termed a "bipotential" progenitor cell. A distinguishing feature of a progenitor cell is that, unlike a stem cell, it has limited proliferative ability and thus does not exhibit self-maintenance. It is committed to a particular path of differentiation and will, under appropriate conditions, eventually differentiate into glia or neurons. The term "precursor cells", as used herein, refers to the progeny of neural stem cells, and thus includes both progenitor cells and daughter neural stem cells.

The mitotically active cells of the hippocampal region can be induced to proliferate and differentiate *in vitro* using the same techniques described in the published PCT applications referenced above. Accordingly, the mitotically active cells of the hippocampal region are referred to herein as "hippocampal stem cells." The hippocampal stem cells and their progeny can be genetically modified using the procedures described in WO 94/16718.

In Vivo Proliferation, Differentiation, and Genetic Modification of Hippocampal Stem Cells and Their Progeny

The hippocampal stem cells can be induced to proliferate and their progeny induced to differentiate *in vivo* by administering to the host, any growth factor(s) or pharmaceutical composition that will induce proliferation and differentiation of the stem cells *in vitro*. Examples of suitable growth factors and pharmaceutical compositions are described in the published PCT applications referenced above. The techniques described in the above-referenced published PCT applications for the proliferation, differentiation, and genetic modification of neural stem cells and their progeny *in vitro* can be adapted to *in vivo* techniques, to induce the proliferation, differentiation, and genetic modification of hippocampal stem cells. Such *in vivo* manipulation and modification of hippocampal stem cells allows for augmentation of normal function as well as replacement of cells lost, due to injury or disease, thus obviating the need for transplanting foreign cells into a patient. Additionally, the cells can be modified or genetically engineered *in vivo* so that they express various biological agents useful in the treatment of neurological disorders.

Administration of growth factors can be done by any method, including injection cannula, transfection of cells with growth hormone-expressing vectors, injection, timed-release apparati which can administer substances at the desired site, and the like. Pharmaceutical compositions can be administered by any method, including injection cannula, injection, oral administration, timed-release apparati and the like. The hippocampal stem cells can be induced to proliferate and differentiate *in vivo* by induction with particular growth factors or pharmaceutical compositions which will induce their proliferation and differentiation. Therefore, this latter method circumvents the problems associated with transplantation and immune reactions to foreign cells. Any growth factor can be used, particularly EGF, TGFα, FGF-1, FGF-2, NGF, and combinations thereof.

Growth factors can be administered in any manner known in the art in which the factors may either pass through or by-pass the blood-brain barrier. Methods for allowing factors to pass through the blood-brain barrier include minimizing the size of the factor, or providing hydrophobic factors which may pass through more easily.

Growth factors may be injected directly into the hippocampal region to induce hippocampal stem cell proliferation, or they may be administered to the lateral ventricle which is near the hippocampal region. The hippocampal stem cell progeny can migrate into regions that have been damaged as a result of injury or disease.

In Vitro Models of CNS Development, Function and Dysfunction, and Methods for Screening Effects of Drugs on Neural Cells

Hippocampal stem cell progeny cultured *in vitro* can be used for the screening of potential neurologically therapeutic compositions using the techniques described in PCT published application no. WO 96/09543.

EXAMPLES

By using ([H^3])-thymidine autoradiography and bromodeoxyuridine (BrdU) immunocytochemistry, we found mitotically active cells throughout the hippocampal region, including Ammon's horn and the dentate gyrus of the adult mouse. The following experiments examine the relative numbers, locations, and ultimate phenotype of newly generated cells in the adult mouse hippocampal region.

EXAMPLE 1:

Labeling of Mitotically Active Cells:

Adult male CD-1 mice (Charles River; 29-33 grams) received a series of intraperitoneal injections of either ([H^3])-thymidine or BrdU using a modified version of the methods set forth by Morshead *et al.*, *(Neuron 13* (1994) ¶. 1071-1082) and in PCT published application no. WO 95/13364, whereby

stem cells were labeled by injecting the mice with BrdU every 2 hours over a period of 48 hours, then sacrificed immediately or allowed to survive for 1, 3, 6, 9, 12, 15 and 24 weeks. Animals were deeply anesthetized with sodium pentobarbital (intraperitoneal injection of Nembutal, 0.1 ml) and perfused transcardially first with 0.9% saline (NaCl) followed by a fixative solution (4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brain was removed from the skull and postfixed overnight at 4°C in the same fixative solution. Following post fixation, brains were cryoprotected in 10% then 20% sucrose in 0.1M phosphate buffered saline (PBS) overnight at 4°C, followed by a solution of 20% sucrose in 0.1M PBS with Tissue-Tek OCT embedding compound at a ratio of 2:1 overnight. Brains were embedded in OCT and frontal sections were cut (14µm) with a MICROM cryostat. Serial sections were mounted on gelatin coated slides and dried at room temperature.

Immunocytochemistry:

Sections processed for BrdU were rinsed in washing solution (0.1 M PBS containing 0.02% sodium azide) for 20 minutes before any immunocytochemistry was performed. The sections were then incubated overnight at room temperature in a solution containing mouse monoclonal antisera to either Calbindin D-28K (Sigma, diluted 1:1000), GFAP (Boehringer Mannheim, diluted 1:200), S-100 (Sigma, diluted 1:500), or NeuN (Gift from R. Mullen, diluted 1:50) all diluted in 0.1M PBS + 0.3% Triton + 10% normal goat serum. The sections were then rinsed in washing solution (3 x 15 minutes) and incubated in anti-mouse IgG CY3 (Jackson, diluted 1:100) for 1 hour. The sections were rinsed again in washing solution (3 x 15 minutes), then incubated in 1.0N HCL for 30 minutes at 60°C to denature the DNA. Following incubation, sections were rinsed in washing solution (3 x 15 minutes), and incubated overnight at room temperature in a primary rat monoclonal antibody (1:50) directed against single-stranded DNA containing BrdU (Seralab). Finally, the sections were rinsed in washing solution (3 x 15 minutes), and incubated for 1 hour in anti-rat IgG FITC (Jackson diluted 1:100

in washing solution) at room temperature then rinsed and coverslipped under FluorSave (Calbiochem).

Autoradiography:

Slides that were prepared for autoradiography were dipped in Kodak NTB-3 (diluted 1:1 in ddH_2O) emulsion and exposed for 14 days at 4°C. Slides were then developed in Dektol (Kodak), rinsed in ddH_2O, fixed in Ektaflo (Kodak), rinsed again, stained for Nissl using Cresyl Violet and coverslipped under Permount.

Photography:

Images were captured on a Zeiss Axioskop 50 microscope and transferred to optical discs using an H.P. Laserscanner. Images were then formatted and printed on Kodak electronic imaging paper.

Results:

Cresyl Violet stained sections of autoradiographs demonstrated that, at day zero, [^{3}H]-thymidine labeled cells were located throughout the dentate gyrus, including the supra- and infra-pyramidal limbs and the hilus/CA4 and crest regions. In addition, [^{3}H]-thymidine labeled cells were present throughout all subfields of Ammon's Horn, including CA1 and CA3. When stained with Neu-N, GFAP or CNPase, no doubled-labeled cells were present immediately following the BrdU labeling protocol, indicating that the [^{3}H]-thymidine labeled cells were not differentiated into neurons or glia.

BrdU labeled cells were also present at day zero, not only in the dentate gyrus but also in all regions of Ammon's Horn and other areas of the hippocampal region, including the subiculum, entorhinal cortex and amygdala, amongst others (Fig. 1). Within the first three weeks the number of BrdU positive cells declined in all regions, most likely as a result of cell death and the diluting out of label by fast cycling cells such as progenitor cells. From 3 to 12 weeks, the

BrdU positive cell count stabilized, suggesting the presence of either slower cycling cells or quiescent cells such as stem cells. A portion of these cells 1) were brightly labeled for BrdU, indicating that they had not diluted their label through repeated cell divisions and 2) did not present antigens typical of differentiated neural cells.

A portion of the BrdU-labeled cells in the infra- and supra-pyramidal limbs and the subgranular zone of the dentate gyrus differentiated into cells with morphological and antigenic characteristics of neural cells. Double-label immunocytochemistry demonstrated that BrdU-labeled cells differentiated into 1) cells with the morphological and antigenic characteristics of astrocytes (GFAP+BrdU; S-100+BrdU) and 2) cells with the morphological and antigenic characteristics of neurons (dual-label NeuN+BrdU; Calbindin+BrdU).

BrdU labeled cells in Ammon's horn differentiated into cells with morphological and antigenic characteristics of neural cells. Double-label immunocytochemistry demonstrated that BrdU labeled cells differentiated into 1) cells with the morphological and antigenic characteristics of astrocytes (dual-label S-100+BrdU) throughout the hippocampal region and 2) cells with the morphological and antigenic characteristics of neurons (dual-label NeuN+BrdU; Calbindin+BrdU) were seen throughout the Ammon's horn, including the stratum pyramidal of CA1. It should be noted that at day zero, no BrdU labeled cells were double labeled, indicating the absence of BrdU labeled differentiated cells. Over the time course of the experiment, undifferentiated BrdU labeled cells were consistently present, indicating the presence of stem cells (Fig. 2).

Figure 1 shows the decrease in the average number of BrdU-labeled cells in the dentate gyrus, CA1 and CA3 from day 0 to 12 weeks. Table 1 shows the quantitative and phenotypic analysis of BrdU-labeled cells over time within the dentate gyrus and Ammon's Horn.

Figure 2 shows the average number of BrdU-labeled cells in the murine hippocampus over 24 weeks. After 3 weeks the number of BrdU-labeled cells stabilizes, indicating a slowly-cycling, relatively quiescent, stem cell population.

TABLE I

Number of BrdU labeled cells
versus total number of cells

Survival Time	BrdU	BrdU + NeuN	BrdU + GFAP	BrdU	BrdU + NeuN	BrdU + GFAP
Day 0	24 ± 0.36	0	0	14 ± 2.4	0	0
Week 3	7 ± 0.81	4 ± 0.74	1 ± 0.99	4 ± 1.3	1 ± 0.93	1 ± 1.3
Week 6	6 ± 1.6	2 ± 1.6	1 ± 0.74	3 ± 1.0	1 ± 0.27	1 ± 1.0
Week 9	6 ± 20	3 ± 1.7	0.5 ± 0.18	3 ± 1.3	0.3 ± 1.9	0 .2 ± 0.27
	Dentate Gyrus			**Ammon's Horn**		

EXAMPLE 2:

Regeneration of Neural Cells in Treatment of Stroke:

Occlusion of the carotid arteries precipitates the occurrence of ischemic damage similar to that which occurs during stroke. Adult Wistar rats, in which the middle cerebral artery has been occluded to produce symptomatic lesions in the hippocampal region are administered a proliferation-inducing growth factor such as EGF using any of the methods disclosed in published PCT application no. WO 95/13364, to the lesioned area to induce proliferation of the mitotically active cells of the hippocampal formation. After a survival period, the animals are tested for behavioral improvements and are then sacrificed and their hippocampal region analyzed and compared with controls.

EXAMPLE 3:

Genetic Modification of the Hippocampal Region:

A replication defective adenoviral (Ad) vector encoding the antiapoptotic gene *bcl-2* under the control of the neuron specific enolase, or a similar promoter, is

delivered to the hippocampal region of a mammal. The Ad vector transduces the cells within the region with high efficiency, regardless of proliferative state, and the vector itself functions episomally, thus avoiding potential problems inherent to random integration of exogenous DNA into the host genome, such as the induction of malignancy.

By introducing such a gene, hippocampal region cells are protected from signals which would normally induce apoptosis such as oxidative stress, growth factor withdrawal and other physiological mechanisms.

EXAMPLE 4:

Administration of substances to stem cells in the Hippocampal Region: Adult CD1 Mice are anesthetized and prepared for administration of EGF to the CA3 area of the hippocampal region. Stereoscopic coordinates are determined for precise placement of the growth factor. The skin over the site is incised and retracted. A hole is drilled in the skull adjacent to the desired site of injection. A small gauge needle, attached to a cannula and syringe containing EGF solution is lowered into position. 2μl of EGF solution (Chiron, 416ng), is administered over a one hour period using a Sage flow rate modulator. The needle is withdrawn and the skull burr holes closed with gel foam. The incision is sutured and the animal allowed to recover. Three weeks later the animals are subjected to the BrdU stem cell labeling paradigm of Example 1. At weekly intervals, animals are sacrificed and the numbers of BrdU labeled cells present in the hippocampal region are compared with control animals which received saline injections in place of EGF.

WHAT IS CLAIMED:

1. A method for the *in vivo* proliferation of a multipotent neural stem cell located in the hippocampal region of a mammal, said method comprising administering at least one growth factor to said hippocampal region to induce the proliferation of said cell.

2. The method of Claim 1 wherein said hippocampal region is Ammon's horn.

3. The method of Claim 1 wherein said at least one growth factor is epidermal growth factor.

4. The method of Claim 3 further comprising administering fibroblast growth factor to said hippocampal region.

5. A method for the *in vivo* genetic modification a CNS precursor cell located in the hippocampal region of a mammal, said method comprising administering genetic material to said hippocampal region to infect said cells, said genetic material being capable of encoding at least one neurological agent.

6. The method of Claim 5 wherein said hippocampal region is Ammon's horn.

7. The method of Claim 5 further comprising administering at least one growth factor to said hippocampal region.

8. The method of Claim 7 wherein said growth factor is epidermal growth factor.

9. The method of Claim 8 further comprising administering fibroblast growth factor to said hippocampal region.

10. The method of Claim 5 wherein said neurological agent is selected from the group consisting of growth factors, growth factor receptors, neurotransmitters, neurotransmitter receptors, neuropeptides, growth factor synthesizing enzymes, and neurotransmitter synthesizing enzymes.

11. A method of treating a neurological disorder of a mammal comprising administering at least a first growth factor to the hippocampal region of said mammal, said growth factor inducing the *in vivo* proliferation of hippocampal stem cells.

12. Use of a growth factor in the manufacture of a medicament for the treatment of a neurological disease, injury or disorder which affects the hippocampal region of the brain.

13. Use according to Claim 12 wherein the medicament is for the treatment of stroke.

14. Use according to Claims 12 or 13 wherein the medicament is adapted for direct administration to the hippocampus.

15. Use according to Claims 12 or 13 wherein the medicament is adapted for direct administration to Ammon's horn.

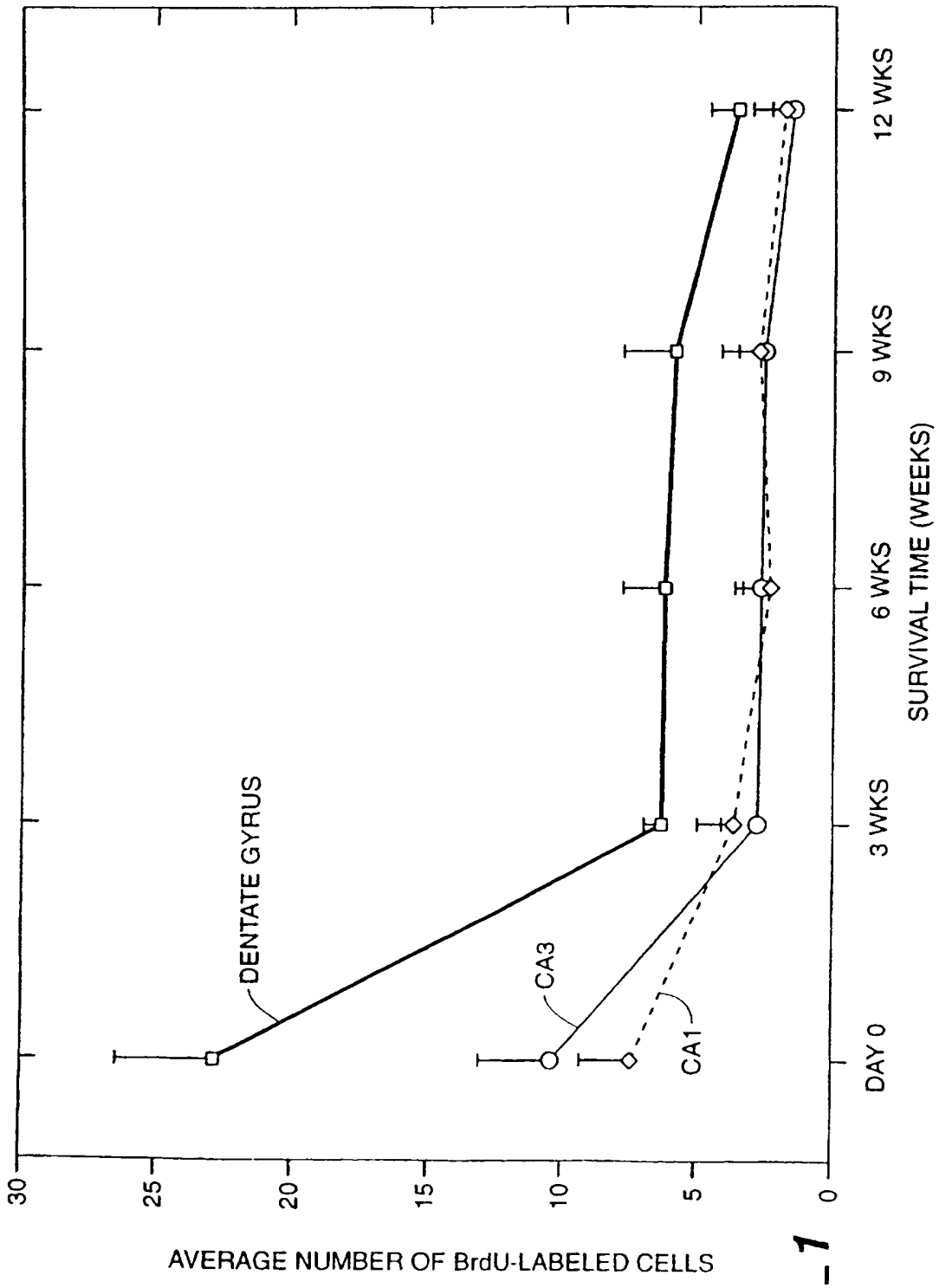
DENTATE GYRUS
CA3
CA1
DAY 0
3 WKS
6 WKS
9 WKS
12 WKS
SURVIVAL TIME (WEEKS)
0
5
10
15
20
25
30
AVERAGE NUMBER OF BrdU-LABELED CELLS

FIG._1

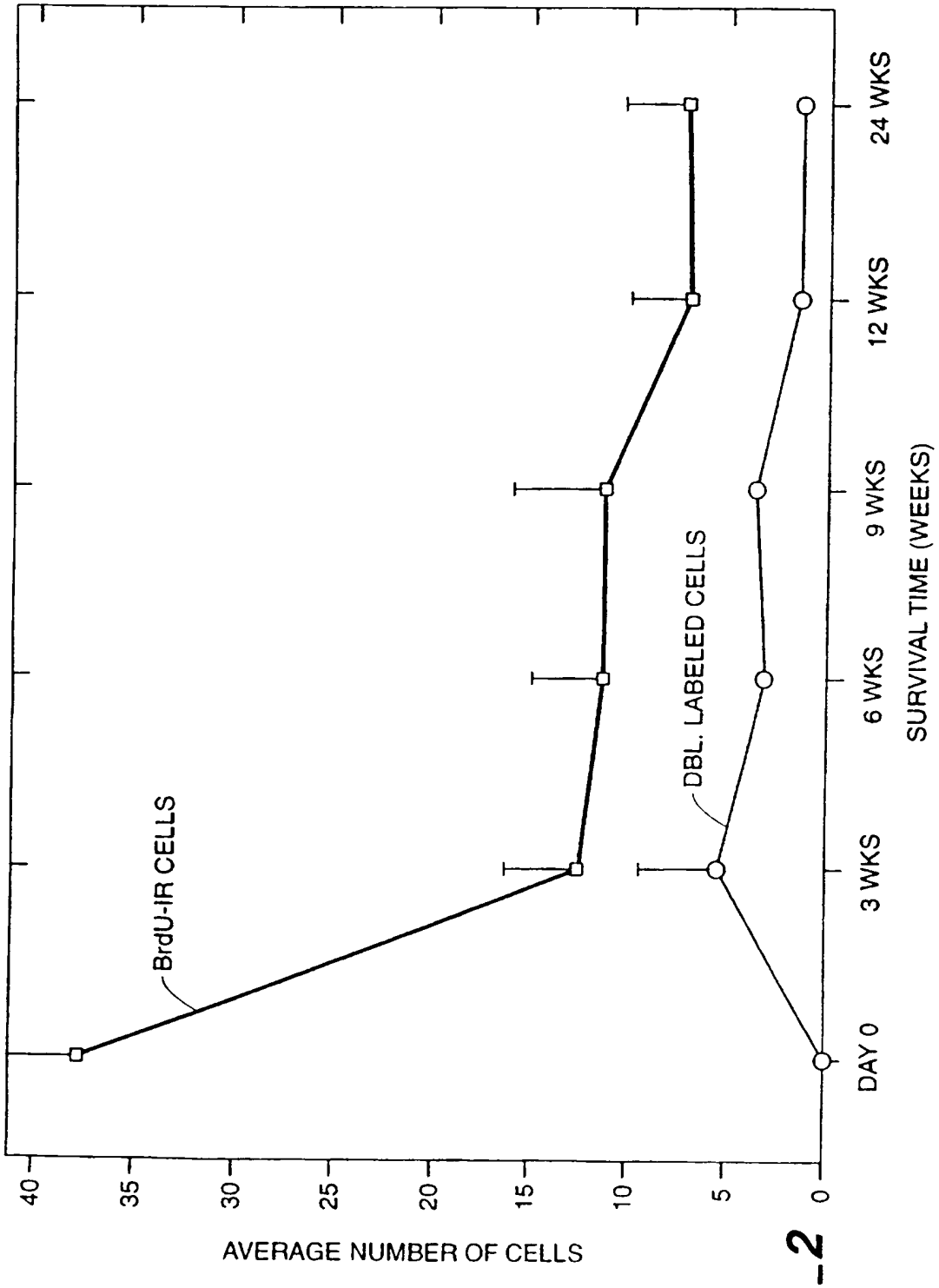
BrdU-IR CELLS
DBL. LABELED CELLS
AVERAGE NUMBER OF CELLS
40
35
30
25
20
15
10
5
0
DAY 0
3 WKS
6 WKS
9 WKS
12 WKS
24 WKS
SURVIVAL TIME (WEEKS)

FIG._2

INTERNATIONAL SEARCH REPORT

Interna .al Application No
PCT/CA 97/00197

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 A61K48/00 //C12N5/00,C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NEURON, vol. 15, no. 1, 1 July 1995, CAMBRIDGE, MA, US, pages 105-114, XP002034680 C. VICARIO-ABEJÓN ET AL.: "FUNCTIONS OF BASIC FIBROBLAST GROWTH FACTOR AND NEUROTRPHINS IN THE DIFFERENTIATION OF HIPPOCAMPAL NEURONS." see page 105, right-hand column, paragraph 3 see page 111, left-hand column, paragraph 2 ---	1-15
Y	WO 95 13364 A (NEUROSPHERES LTD.) 18 May 1995 cited in the application see claims 1-15,23-29,36-59 --- -/--	1-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1997	18. 07. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Ryckebosch, A

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 97/00197

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, December 1995, WASHINGTON US, pages 11879-11883, XP002034681 F.H. GAGE ET AL.: "SURVIVAL AND DIFFERENTIATION OF ADULT NEURONAL PROGENITOR CELLS TRANSPLANTED TO THE ADULT BRAIN" see page 11883 ---	1-15
P,Y	MOLECULAR AND CELLULAR NEUROSCIENCE, vol. 8, no. 6, 1997, SAN DIEGO, CA, US, pages 389-404, XP002034682 T.D. PALMER ET AL.: "THE ADULT RAT HIPPOCAMPUS CONTAINS PRIMORDIAL NEURAL STEM CELLS." see the whole document -----	1-15

1

INTERNATIONAL SEARCH REPORT

:national application No.
PCT/CA 97/00197

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1 - 11
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos..
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. nal Application No
PCT/CA 97/00197

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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